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(54) Title: SOLID STATE CULTURE OF WHITE ROT FUNGI

(57) Abstract

White-rot fungi are grown on a sugar beet pulp substrate. By-products of fungal growth, such as lignin-degrading enzymes, can be recovered from the culture. The culture or enzymes recovered from the culture can be used to degrade aromatic compounds in bioremediation procedures.

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"Solid State Culture of White Rot Fungi"

Background of the Invention

Enzymes for degrading aromatic compounds have potential commercial application in the pulp and paper industry, the production of fuels and chemicals from lignocellulose, the enhancement of livestock feeds, and the bioremediation of aromatic hazardous wastes.

Lignin is a complex polymer of phenyl propanoid units with a variety of interunit linkages forming a nonlinear, random structure. Lignin comprises 10-35% of the dry weight of lignocellulose-rich materials such as wood, straw, and corn stover. Lignin is resistant to biological destruction, although it is enzymatically degraded by various higher order fungi. In nature, the basidiomycetes that cause white-rot wood decay are major degraders of lignocellulose. White-rot fungi oxidize lignin completely to carbon dioxide. Extracellular enzyme complexes secreted by these fungi catalize oxidative reactions of the lignin structure. White-rot fungi have also been shown to oxidize and degrade a wide range of other aromatic structures including a variety of man-made, toxic aromatic compounds. The term "white-rot fungi" as used herein is intended to include fungi having enzymes capable of oxidizing and thereby degrading aromatic compounds.

There are an estimated 1700 species of white-rot fungi. However, research on enzymatic lignin degradation has concentrated on one organism: Phanerochaete chrysosporium. Lignin-degrading enzymes from this organism have been purified and characterized. A large volume of research literature describes processes for growing P. chrysosporium in liquid media for lignin degradation or production of lignin-degrading enzymes. The conventional production of lignin-degrading enzymes in liquid media occurs during secondary metabolism and is initiated by nitrogen or glucose starvation. For instance, in U.S. Patent 4,554,075, Chang et al. describe a process for growing white-rot fungi by carrying growth into secondary metabolism wherein nitrogen starvation occurs. See also Ming Tien in an article in CRC Critical Reviews in Microbiology, titled "Properties of Ligninase From Phanerochaete Chrysosporium and Their Possible Applications", Volume 15, Issue 2 (1987) at p. 143 and U.S. Patent 4,891,230 to Aust et al.

The slow growth rates and low cell mass production associated with starved cultures results in long growth times and low yields thus making this impractical for commercially producing enzymes for pretreating wood pulp in paper making processes, for in situ treatment of toxic waste, or for enhancing lignocellulose for livestock feed. Tien notes on page 144 in the same article listed above that scale-up from liquid culture grown in flasks has proven difficult.

To overcome the low cell mass production, the art has suggested growing several species of white-rot fungi using solid culture media in solid state reactors. In these instances, the fungus grows on a substrate of moist solid lignocellulose-containing materials. Straw, several types of wood, and milled corn cob have been disclosed as substrates in the literature. These materials have been selected as culture substrates primarily because they are typical of the materials degraded by the white-rot fungi in nature. They have a relatively high lignin content of 10-35%, low nitrogen levels, and limited access to cellulose as a carbon source. White-rot fungi can be grown in such solid-state cultures, but obtaining lignin-degrading enzymes in cell and solids free extracts of such cultures has proved an elusive task as the enzyme activity remains bound to the substrate.

Several patents as well as other literature disclose processes for preparing ligninase in solid cultures including U.S. Patent 4,711,787 to Odakra, which describes using okra as a substrate for the production of livestock feed. Rolz, et al., in an article in Applied Microbiology and Biotechnology titled, "White-Rot Fungal Growth on Sugarcane Lignocellulosic Residue", Volume 25 (1987) pp. 535-541, report using sugarcane residue as a substrate. In U.S. Patent 4,891,320, Aust et al. list as typical materials used to grow white-rot fungi for use in degradation of aromatic compounds shredded paper, wood shavings, sawdust, corn cobs, and humus. None of these references discloses the production of

enzymes during the primary metabolic growth phase or the production of cell-free extracts of the culture containing lignin-degrading enzymes.

It is believed that the reason why extracting cell-free enzymes is difficult in conventional solid state processes for producing enzymes is that the enzymes are absorbed into the lignocellulosic substrate materials. Thus, when using substrates of the type normally associated in nature with white-rot fungi, lignin-degrading enzymes are difficult to extract or purify in active form. These substrates typically have a high lignin content and low protein content. On the other hand, small amounts of cell-free enzymes are present in liquid cultures, presumably because there are no surfaces for enzyme absorption.

Both liquid and solid substrate cultures of white-rot fungi have been the subject of at least 15 years of intensive research in numerous laboratories, as evidenced by the volume of research literature and patents granted in this field. However, the problems of producing enzymes during the primary metabolic growth phase, of producing cell-free enzymes from solid culture and of producing lignin-degrading enzyme preparations with commercially useful enzyme concentrations remain unsolved.

Summary of Invention

This invention pertains to a novel composition of matter comprising a solid state culture of white-rot fungus in a mixture with a substrate comprising as an important ingredient sugar beet pulp. This invention also pertains to the process for growing white-rot fungus in solid state culture using sugar beet pulp and the use of the fungal culture to degrade aromatic compounds such as lignin or other aromatic organic pollutants. The culture also can be used for production of by-products of fungal growth such as lignin-degrading enzymes. The culture advantageously permits the production of lignin-degrading enzymes by the white-rot fungi during the primary metabolic growth phase of the fungus rather than during secondary metabolism. Furthermore, the lignin-degrading enzymes can be separated easily from the substrate material for the production of cell-free enzymes preparations.

The culture is prepared by growing white-rot fungus under growth-supportive conditions on a substrate comprising sugar beet pulp. An inoculum culture of white-rot fungus is prepared for inoculating the substrate. Water and nutrients are added. A substrate of sugar beet pulp is prepared typically by sterilizing the substrate as by autoclaving and then cooling the substrate. The substrate is inoculated with the prepared inoculum. The inoculated substrate is then placed in a solid state reactor for growing fungi, and the mixture is aerated to enhance growth. Nonlimiting examples of white-rot fungi that can be grown in the substrate include species from the genera Phanerochaete, Phlebia, Trametes, Pleurotus, and Bjerkandera.

At the conclusion of the growing period, the culture can be used without further processing. For example, the culture can be used in bioremediation processes to degrade aromatic organic pollutants (e.g. polynuclear aromatic hydrocarbons and chlorinated aromatic compounds) in a soil or water mass. Alternatively, extracts rich in lignin-degrading enzymes may be separated from the substrate.

For production of by-product of fungal growth, one can isolate by-products from the culture after an appropriate growth period. For example, the substrate can be washed with water to bring aqueous-soluble enzymes such as ligninases into solution. The lignin-degrading enzymes can be recovered separate from the substrate using this process. The enzyme-rich solution can be centrifuged and filtered to provide a cell free liquid enzyme preparation containing lignin-degrading enzymes that have been removed from the substrate.

The growth of white-rot fungi on sugar beet pulp substrate results in the ability to produce lignin-degrading enzymes during the primary metabolic growth phase of the fungus when an abundance of nutrients are available and growth rate is optimal rather than in secondary metabolism with limited nitrogen or carbon. The ability to produce lignin-degrading enzymes commercially during the primary metabolic growth phase and to produce cell free lignin-degrading enzymes is an advantage of this invention over conventional solid state or liquid culture process used to produce these enzymes using white-rot fungi.

Brief Description of the Figures

Figures 1A and 1B are gas chromatograms of polychlorinated biphenyl compounds in control and fungus-treated samples of soil.

Figures 2A and 2B are the same for a different experiment.

Detailed Description of the Invention

Sugar beet pulp is used as the substrate material for fungal growth in accordance with this invention. Sugar beet pulp is produced in large amounts and is readily available for high-volume, commercial applications for growing white-rot fungi.

Sugar beet pulp has not been reported as a natural substrate for white-rot fungi. It has a relatively low lignin content of 1% to 3%. White-rot fungi occurs naturally as decay organisms on woody materials with high lignin content such as okra, sugarcane, shredded paper, wood shavings, sawdust, corn cobs and humus. These materials have been used in conventional production of lignin-degrading enzymes.

Sugar beet pulp contains 8-10% protein and up to 5% residual sucrose and is not a carbon and nitrogen limited substrate. Yet, white-rot fungi produce lignin-degrading enzymes when grown on sugar beet pulp during the primary metabolic growth phase. Lignin-degrading enzymes are produced by white-rot fungi when grown on sugar beet pulp supplemented with glucose and the additional nitrogen sources peptone (a soluble protein hydrolysate) and yeast extract. This result is unexpected because production of these enzymes using conventional processes typically occurs only with nitrogen or carbon starvation during secondary metabolism.

Sugar beet pulp is a byproduct of the processing of sugar beets for sugar (sucrose). In a typical process, sugar beets are sliced and extracted with hot water to recover the sugar. Sugar beet pulp is the residue of sugar beets remaining after the extraction process. In most sugar beet processing plants, the sugar beet pulp is dried and sold as cattle feed. Sugar beet pulp is composed of the following constituents with the typical proportions shown as a percentage on a dry weight basis.

Mean chemical composition of raw sugar beet pulp

Components	Raw Pulp
Dry matter	91.5
Total Nitrogen (x 6.25)	10.8
Protein Nitrogen (x 6.25)	9.0
Ashes	4.3
Organic Matter	95.7
ADF[a]	23.3
NDF[b]	51.9
Lignin	1.0
Cellulose (ADF-Lignin)	22.3
Hemicellulose (NDF-ADF)	28.6
Gross Energy (kcal/kg dry matter)	4217

[a] This is acid detergent fiber.
[b] This is neutral detergent fiber.

* A. Duranl and D. Cherau (1988); "A New Pilot Reactor for Solid State Fermentation: Application to the Protein Enrichment of Sugar Beet Pulp"; Biotechnology and Bioengineering, Vol. 31, pp 476-486.

Particles of sugar beet pulp are typically 0.5 to 1 cm in the largest dimension and irregularly shaped.

Sugar beet pulp can be prepared for use as a solid culture substrate as follows. Dry sugar beet pulp is moistened with one of a number of standard nutrient solutions supportive of fungal growth and

then sterilized by autoclaving, e.g., at 125°C, 15 psi for 20 minutes. Other generally accepted methods for sterilization can be used involving different temperatures, pressures, and durations as long as the sugar beet pulp is sterilized before inoculation. The sugar beet pulp is then cooled to between 20-40°C.

An inoculum of white-rot fungi is then aseptically and thoroughly mixed with the cooled sugar beet substrate. The inoculum can be prepared in any conventional manner such as by first selecting a pure culture of a white-rot fungus and maintaining this fungus on nutrient agar slants. Next, the culture on the agar slants is transferred to either a liquid or solid media and grown at 20-40°C. The media selected varies somewhat depending upon which organism is selected for growth. If a liquid media is selected for growing the inoculum, the liquid inoculum media should contain glucose, a nitrogen source, and nutrient salts. Liquid cultures can be held stationary or agitated during the culture growth phase. If a solid media is selected for growing the inoculum, either sterilized sugar beet pulp, prepared as described above, or other known materials can be used as a substrate. Generally, sufficient inoculum culture is grown to provide approximately 1-20% by volume of the mass of substrate to be inoculated.

According to the present invention, the inoculated sugar beet pulp comprises a solid state culture characterized by a solid phase of particles of sugar beet pulp, an aqueous phase sorbed into the particles of the pulp and a gas phase in the interparticle spaces. Moisture content of the sugar beet pulp is 40 to 80%, typically 66% by weight.

Optionally, 2-10% sterilized straw can also be added to the sugar beet pulp. Straw may be added before or, more typically, after the beet pulp is wetted. The straw improves the physical characteristics of the solid culture by increasing the volume and maintaining integrity of interparticle spaces resulting in improved aeration, temperature control, and moisture control.

The fungus grows on the surface of, and penetrates into, the particles of sugar beet pulp.

The inoculated substrate is placed in a vessel designed as a solid culture reactor or in a trench or pile. The shape and dimensions of the vessel used as the solid culture reactor may be varied widely. In one currently developed embodiment, the inoculated substrate is placed in cylindrical or rectangular vessel in a bed approximately 70 cm deep. The vessel is designed so that air at controlled temperature and humidity can be circulated through the bed and appropriate means are provided for this.

In a solid state reactor, the temperature, nutrients, aeration rate, and growing period can be varied to regulate the metabolic rate of the fungus. Metabolic conditions also can determine the specific types of lignin-degrading enzymes produced by the fungus. Typically, the temperature of the substrate is maintained between 20-40°C depending on the organism and enzyme preparation being produced. A nutrient solution may be added to the substrate as necessary to maintain primary metabolic growth phase. Sufficient conventional nutrient solution is provided during the growing period to prevent nitrogen or carbon starvation or secondary metabolism.

An atmosphere of air, or an artificially created atmosphere having an oxygen concentration of 7-100%, is circulated through the substrate during the growing period. An aeration rate of between .05 to 20 unit volumes of air per minute per unit volume of substrate may be used. The aeration atmosphere preferably is maintained between 70-99% relative humidity. The relative humidity typically is varied to maintain the absorbed water content of the substrate between about 40-80% initially, and then between about 60-80% at the end of the growing period, with 66-72% being typical. The growing period of the culture is varied from 4 to 30 days, depending on the identity of the organism and the type of enzyme to be produced.

At the completion of the growing period, the culture comprises a fungal cell mass, unutilized culture substrate, and extracellular enzymes. For some applications, particularly in situ degradation of toxic wastes, the whole wet culture may be used without further processing by merely turning the culture into the soil.

The method of this invention can be used to degrade polyaromatic hydrocarbons and polyhalogenated aromatic compounds such as polyhalogenated biphenyl compounds in a variety of materials. The method can be used in the bioremediation of soils, aquatic sediments, gravels or other solid materials contaminated with polyhalogenated biphenyl compounds.

For bioremediation of soils, whole wet culture is spread on the soil surface and mixed to thoroughly disperse the particles of white-rot fungus, sugar beet pulp culture through the soil. In laboratory experiments mixing can be accomplished by stirring.

In many contaminated sites, contaminants have spilled on the surface and contamination is confined to the top 25-50 cm of soil. In these cases the fungus, sugar beet pulp culture is spread on the soil surface and mixed using tilling equipment such as a rototiller, tractor and plow, etc. The methods and implements to accomplish mixing may vary if uniform dispersion of white-rot fungus culture through the soil can be achieved. Where contamination extends too deep for effective mixing or is not accessible to direct mixing as in the case of underwater sediments, the material to be treated may be excavated and mixed with the white-rot fungus, sugar beet pulp culture. The mixture can then be spread in windrows or lifts on a surface or placed in a container such as a lined trench or tank.

The volume of white-rot fungus, sugar beet pulp culture added to a given volume of soil varies with soil characteristics (such as pH and density) concentration of polyhalogenated biphenyls and treatment time. For low concentrations of contaminant generally 100 ppm or less, one application of a volume of fungus culture equal to 25% of the volume of soil may be sufficient to achieve the desired level of remediation. With high concentrations of contaminant or for more rapid degradation, up to 150% volume fungus culture to volume of soil may be necessary. Alternatively, several additions of 25% fungus culture volume at 10 to 20 day intervals may be the most effective.

Moisture content of the mixture of soil and fungus culture is typically maintained at 40-60%, though this may vary depending on water capacity of the soil and volume of fungus culture used. Temperature for treatment must be within a range supportive of growth and metabolism of the species of white-rot fungus being introduced. Generally this is in the range of 10 to 40°C. Time required to achieve a specific level of degradation will vary with contaminant, its concentration, soil characteristic, volume of culture, temperature and moisture. Significant degradation of polyhalogenated biphenyls may be achieved in a few days up to several months.

In addition to the use of whole, wet culture for remediation, cultures may be processed by forming a slurry that can be pumped and mixed more easily in some types of materials. Cultures may also be dried for improved storage and transportation and rehydrated immediately prior to application.

To produce a cell-free liquid enzyme preparation containing lignin-degrading enzymes, one can extract the culture by mixing it with water. Alternatively, water together with conventional, biologically compatible detergents, such as TWEEN 80, may be used as an extractant. A cell-free solution containing lignin-degrading enzymes can be produced by mixing the culture with the extractant, then centrifuging and filtering to remove all cells and solids (with, for example a 0.8 micron filter).

The sugar beet pulp substrate is capable of sustaining growth of a variety of white-rot fungi to induce production of at least four types of enzymes, namely, peroxidases, manganese peroxidases, oxidases and laccases. To determine the nature of the enzymes present in various extracts, conventional assay procedures such as those based on enzymatic oxidation of compounds such as phenol red, veratryl alcohol, vanillylacetone and anis alcohol with and without the presence of hydrogen peroxide or oxygen or manganese are used.

Assays of peroxidase are based on oxidation of phenol red or veratryl alcohol in the presence of hydrogen peroxide. See e.g., Tien, M. (1987) Critical Review in Microbiology 15(2):144; Farrell, R., U.S. Patent No. 4,687,741; Kuwahare, M. et al. (1984) FEBS Letters 169(2):247-250; Walder, R. et al. (1988) Applied Microbiology and Biotechnology 29:400-407. Assays for manganese peroxidase measure oxidation of phenol red, veratryl alcohol or vanillacetone with the presence of both hydrogen peroxide and manganese. See Kuwahare, M. et al. and Walder, R. et al., supra; Bonnarme, P. and Jefferies, T.W. (1990) Applied and Environmental Microbiology 56(1):210-217. Assays for oxidase are based on oxidation of veratryl alcohol or anis alcohol with the presence of oxygen. See Muheim, A. et al. Enzyme and Microbial Technology; Walder, R. et al., supra. Assays of laccase activity is based on oxidation of phenol red or 2,6-dimethoxy phenol in the absence of hydrogen peroxide and manganese. See Kuwahare, M. et al. and Walder, R. et al., supra; Haars, A. and Huttermann, A. (1980) Archives of Microbiology 125:233-237.

As illustrated in the examples below, culture extracts grown by the processes of this invention have been assayed using each of these procedures. The presence or absence of hydrogen peroxide, manganese, and oxygen in the enzyme reaction provides a basis for distinguishing the different types of activities.

It is an important feature of the invention that all of these different types of enzymes can be produced. Different commercial applications may require specific types or combinations of these types of enzyme activities. Furthermore, the different types of enzymes produced by various white-rot fungi grown by this process, differ in substrate specificity, pH optima, buffer requirements and stability. These differences may confer relative advantages of one organism and or one type of enzyme in specific commercial applications.

The invention is illustrated further by the following examples. All percentages are by weight and all inoculum mixture proportions are by volume unless otherwise noted.

Example 1

Production of Mn Peroxidase using *P. chrysosporium*

P. chrysosporium obtained from the USDA Forest Products Laboratory (strain BKM) was grown without agitation for 10 days at 25°C in a high-nitrogen, stationary-liquid medium composed of 10 g/l glucose, 5 g/l peptone and 3 g/l yeast extract (Difco). This liquid culture was used as an inoculum culture for the

solid culture medium. The solid culture medium consisted of dried sugar beet pulp wetted to 66% moisture with a nutrient solution disclosed in Table 1:

Table 1

TYPICAL NUTRIENT SOLUTION USED

Substance	g/l	Substance	g/l
Glucose	10.0	$CaCl_2.2H_2O$	.03
$NH_4H_2PO_4$	.05	Trace Elements	5 ml stock solution
KH_2PO_4	1.0	Veratryl Alcohol	0 or .14
$MgSO_4.7H_2O$	1.0	Peptone	.05
		Yeast extract	.05

The wetted sugar beet pulp was autoclaved at 120°C, 15 psi, for 20 minutes, cooled, and inoculated at the rate of 10 ml inoculum cultures per 100 ml of sugar beet pulp substrate. The solid culture was incubated for 5 days at 28°C with an air flow of .2 volume of air per volume of culture per minute with the air at 90% relative humidity. At 5 days, the culture was extracted by adding 3 volumes of water per one part wet weight of whole culture, blended for one minute, centrifuged, and passed through a 0.8 micron filter to produce a cell and solids-free, liquid enzyme preparation. The extracted enzyme preparation was assayed using the phenol red and vanillylacetone assays. In the presence of both hydrogen peroxide and manganese, activity was 80 Phenol Red Units per ml as

assessed by the phenol red assay and .92 International Units per ml by vanillylacetone assay. Mn peroxidase was the only activity detected in this preparation. "Phenol Red Units" may be defined as a 0.1 absorbance change in the optical density of a standardized assay in 30 minutes. An "International Unit" may be defined as the production of 1 μmole of reaction product per minute using conventional assay techniques such as those exploiting veratryl alcohol, anis alcohol, and vanillylacetone.

Example 2

Production of Mn peroxidase and laccase using *P. chrysosporium*

P. chrysosporium was grown under the conditions described in Example 1, except that the inoculum volume was 5%, and the dry sugar beet pulp was wetted to 66% moisture with a nutrient solution including 10 g/l glucose, 5 g/l peptone, and 3 g/l yeast extract. Cultures were grown for 14 days and extracted with two volumes of water per 1 volume wet weight culture.

Extracts which were assayed with phenol red contained 62 Phenol Red Units per ml of Mn peroxidase activity and 27 Phenol Red Units per ml of laccase activity.

Example 3

Pilot scale production of Mn peroxidase

Cultures were grown under conditions described in Example 1 except that 5% by weight (dry basis) milled straw was added to the sugar beet pulp preparation. Cultures were grown in a 20 liter vessel with a substrate bed depth of 70 cm, aerated with 1 volume air per volume of culture per minute at 27-30°C. Extracts of cultures harvested at 10 days showed Mn peroxidase activity at 56 Phenol Red Units.

Example 4

Production of peroxidase, Mn peroxidase and laccase/oxidase using T. versicolor

An inoculum culture of Trametes versicolor (ATCC 48424) was grown in stationary culture in the salts solution of Example 1 at 27°C for 7 days. The inoculum culture was used to inoculate (5% v/v) a series of identical solid cultures composed of sugar beet pulp wetted to 66% moisture with the high nitrogen solution of Example 2. Each of the cultures were incubated at 27°C with an air flow of .2 vol/vol culture per minute at 90%RH. These identical cultures were extracted in 4 volumes of water at different time intervals and assayed for enzyme activity using phenol red. Results are shown below:

Culture Time in Days	Phenol Red Units of Mn Peroxidase	Phenol Red Units of Peroxidase	Phenol Red Units of Laccase/Oxidase
10	20	18	0
17	25	17	44
24	86	37	107

Laccase/oxidase activity is oxidation of phenol red without hydrogen peroxide or manganese. Assay techniques used in this example do not distinguish between laccase and oxidase type activities.

An additional type of enzyme activity may be produced by growing *Trametes versicolor* according to the method of this example. This is an activity that oxidizes phenol red in the presence of manganese but without hydrogen peroxide. This activity is present in 10 day cultures at 12 Phenol Red Units per ml extract and in 17 day cultures with 47 Phenol Red Units per ml.

Example 5

Production of Mn peroxidase and peroxidase using *T. versicolor*

Cultures were grown and extracted under the conditions described in Example 4 except that the inoculum nutrient solution was 10 g/l glucose, 5 g/l peptone and 3 g/l yeast extracts instead of the salts solution. At 10 days culture the extracts contained 22 Phenol Red Units of Mn peroxidase activity and 33 Phenol Red Units of peroxidase activity per ml. Extracts showed no laccase or oxidase activities.

Example 6

Pilot scale production of Mn peroxidase and peroxidase using *T. versicolor*

Cultures were grown under conditions described in Example 4 except that 3% by weight (dry basis) milled straw was added to the sugar beet pulp preparation. Cultures were grown in a 20 liter vessel with substrate bed depth of 70 cm, aerated with 1 volume of air per volume of culture per minute. Temperature was maintained at 27-30°C. Extracts of cultures were made at 10 days with 2 volumes of water per volume wet weight of culture. Extracts contained 37 Phenol Red Units per ml Mn peroxidase, 72 Phenol Red Units per ml peroxidase, and 27 Phenol Red Units per ml laccase/oxidase activity by phenol red assay.

Example 7

Production of Mn peroxidase using *P. tremellosus*

Inoculum cultures of *Phlebia tremellosus* were grown at 27°C for 14 days in unagitated high nitrogen liquid media. Sugar beet pulp was wetted to 57% moisture with the nutrient solution shown below:

	grams/liters
$NH_4H_2PO_4$	.2
KH_2PO_4	2.72
$Mg\ SO_4 .7H_2O$	.5
$CaCl_2$	.1
Yeast Extract	.05
Thiamine	.001
Veratryl Alcohol	.10
Trace Elements	5.0ml
Glucose	10 g/l

Three cultures were grown in this experiment. The first with the nutrient solution, the second with the nutrient solution supplemented with an additional 20 g/l glucose, and the third supplemented with an additional 20 g/l glucose plus 5 g/l peptone and 3 g/l yeast extract. Cultures were grown for 12 days, at 27°C, with 0.2 volumes of 90% RH air per volume of culture per minute. Cultures were extracted with 2 volumes of water per volume wet weight culture. Extracts of all three cultures contained high levels of Mn peroxidase activity in phenol red assay as shown below:

Culture Medium	Phenol Red Units of Mn Peroxidase
Salts	10
Salts plus glucose	25
Salts plus glucose, peptone and yeast extract	78

Mn peroxidase was produced regardless of glucose or nitrogen concentration and was the only activity detected.

Example 8

Production of peroxidase and Mn peroxidase using B. adusta

Inoculum cultures of Bjerkandera adusta (CBS 595.78) were grown for four days at 28°C in an agitated nutrient solution comprising 10 g/l glucose, 5 g/l peptone and 3 g/l yeast extract. Sugar beet pulp was wetted to 70% moisture with the same high nitrogen media and inoculated at 10% v/v with the inoculum culture. Inoculated sugar beet pulp was incubated for 10 days at 27°C with an air flow of 2 volumes of air per volume of culture per minute with the air at approximately 90% relative humidity.

After 10 days, extracts were made with the addition of two volumes of water per volume wet weight culture by the method of Example 1. Extracts were assayed for peroxidase, Mn peroxidase and oxidase using phenol red. The extracts contained 47 Phenol Red Units per ml Mn peroxidase and 45 Phenol Red Units per ml peroxidase. Extracts showed no oxidase or laccase activity.

Example 9

Production of Mn peroxidase using B. adusta

B. adusta was grown, extracted, and assayed as described in Example 8, except cultures were grown at 20°C. Extracts were made at 14 days culture time. Assays showed 101 Phenol Red Units per ml Mn peroxidase. Extracts also showed manganese peroxidase

activity as assessed by veratryl alcohol assay at .43 International Units/ml. Extracts showed no oxidase or laccase activity.

Example 10

Production of peroxidase using _B. adusta_

B. adusta was grown and extracted as described in Example 8 except that extracts were made at 12 days culture time. Extracts contained 98 Phenol Red Units per ml peroxidase activity by phenol red assay. Extracts showed no Mn peroxidase, oxidase or laccase activity.

Example 11

Production of aryl alcohol oxidase using _B. adusta_

Bierkandera adusta was grown under the same conditions as Example 8, except that the sugar beet pulp preparation was wetted with water and the culture grown for 14 days at 30°C. Aqueous extracts contained aryl alcohol oxidase as demonstrated by assay using anis alcohol and veratryl alcohol.

Extracts showed no manganese or hydrogen peroxide dependent activity in these assays. Oxidase activity was .667 International Units per ml of extract by anis alcohol assay and .30 International Units per ml by veratryl alcohol assay.

Example 12

Production of peroxidase using B. adusta

Bjerkandera adusta was grown under the same conditions as Example 8 except that 5% milled barley straw was added to the sugar beet pulp and the culture was grown in a 20 liter vessel aerated with 1 volume of air per volume of culture per minute in a 70 cm deep substrate bed. Extracts of cultures at 10 days showed peroxidase activity assayed using phenol red. Activity was 56.5 Phenol Red Units per ml.

Example 13

Degradation of chlorinated herbicides using cultures of B. adusta grown on sugar beet pulp

Soil contaminated with chlorinated herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) was decontaminated using a culture of B. adusta grown on sugar beet pulp. The contaminated site is in Joliet, Montana. Contaminated soil is under the raised wooden floor of a building used to store herbicides. The building and the floor prevented any photodegradation of the chlorinated compounds from taking place.

Inoculum cultures of B. adusta were produced as described in example 8 and used to inoculate 5 liter volumes of sugar beet pulp substrate prepared as in example 8. Inoculated substrate was placed in 10

liter vessels in a 10 cm deep bed and incubated for 10 days at 22-25°C with a flow of 1 volume of air per volume per volume of culture per minute at approximately 10% RH.

After 10 days, three separate cultures were pooled, transported to the site and mixed with soil. A volume of culture equal to 18% of the volume of soil was used in Plot 1 while a volume of culture equal to 4% of the soil was used in Plot 2. Each plot was approximately one meter square with contamination extending down one meter. The concentration of contaminants was different in the two plots. Soil was treated to a depth of approximately 13 cm through rototilling. Treated soil was sprayed lightly with water as necessary to maintain soil moisture. A third plot was used as a control plot. No fungus was applied to this plot.

Samples of contaminated soil were removed from the two treatment plots prior to addition of the fungus. A soil sample was also taken from the control plot at this time. Final soil samples were taken 74 days later. Soil samples were analyzed for chlorinated herbicides by an EPA approved laboratory using standard EPA method 8150. Laboratory results are shown in the table below:

CONCENTRATION IN PPM

Plot ID	Contaminant	Initial Conc.	Final Conc.
Plot 1	2,4-D	1,100.00	680.0
Plot 2	2,4-D	680.00	4.4
Control	2,4-D	320.00	370.0
Plot 1	2,4,5-T	12.0	13.0
Plot 2	2,4,5-T	.1	1.3
Control	2,4,5-T	370.0	390.0

Example 14

Degradation of chlorinated herbicides using cultures of P. chrysosporium grown on sugar beet pulp

Soil contaminated with chlorinated herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) was decontaminated using a culture of P. chrysosporium grown on sugar beet pulp. Chlorinated dioxins were also present in the soil and most likely were a by-product of the 2,4,5-T manufacture. The contaminated site is in Joliet, Montana. Contaminated soil is under the raised wooden floor of a building used to store herbicides. The building and the floor prevented any photodegradation of the chlorinated compounds from taking place.

Inoculum cultures of P. chrysosporium were produced as described in example 2 and used to inoculate 5 liter volumes of sugar beet pulp substrate prepared as in example 1. Inoculated substrate was placed in 10 liter vessels in a 10 cm deep bed and incubated for 6 days at 22-25°C with a flow of 1 volume of air per volume per volume of culture per minute at approximately 10% RH.

After 6 days, two separate cultures were pooled, transported to the site and mixed with soil. A volume of culture equal to 18% of the volume of soil was used in Plot 3. The plot was approximately one meter square with contamination extending down one meter. Soil was treated to a depth of approximately 13 cm through rototilling. Treated soil was sprayed lightly with water as necessary to maintain soil moisture. An untreated plot was used as a control plot.

Samples of contaminated soil were removed from the treated plot prior to addition of the fungus. A soil sample was also taken from the control plot at this time. Final soil samples were taken 74 days later. Soil samples were analyzed for chlorinated herbicides and dioxins using EPA approved laboratories using standard EPA methods. Herbicides were analyzed for using Method 8150 while dioxins were analyzed for using an EPA approved method incorporating Low Resolution Mass Spectrometry. Laboratory results are shown in the following tables:

CHLORINATED HERBICIDES
Concentration in ppm

Plot ID	Contaminant	Initial Conc.	Final Conc.
Plot 3	2,4-D	1,100	17
Control	2,4-D	320	340
Plot 3	2,4,5-T	12	0.26
Control	2,4,5-T	370	390

Site Demonstration - Dioxin Results

Dioxin Compound	Starting Conc.	Final Conc.	Detection Limit
TCDD (total)	0.16 ppb	N.D.	.090
PeCDD	<0.10	N.D.	.090
HxCDD	<0.13	N.D.	.012
HpCDD	0.88	0.079	.021

Example 15

Degradation of polynuclear aromatic hydrocarbons (PAH) in creosote contaminated soils using cultures of *P. chrysosporium* grown on sugar beet pulp

Cultures of *P. chrysosporium* grown on sugar beet pulp were prepared as described in Example 1. At the time the cultures were mixed with the contaminated soil, the cultures contained 30.7 units per gram wet weight of Mn Peroxidase activity assayed using phenol red.

The soil was obtained from a site contaminated with creosote. 50g soil samples were placed in one liter bottles. Fungal cultures were mixed in with the soil samples at 25, 50, and 75% volume of fungus to volume of soil. The soil samples were incubated for either 30 or 45 days at room temperature. After either 30 or 45 days, depending on the sample, the entire sample of soil and fungal culture was extracted and analyzed. EPA method 8100 for analysis of PAH was used. Concentrations of the four principal PAH compounds are shown in the following table:

Constituent	Untreated	25%	50%	75%	Time
Naphthalene*	2500 ppm	50 ppm	50 ppm	50 ppm	30 d.
Acetnaphthene	65000	29000	20000	20000	30
Fluorene	42000	26000	16000	10000	30
Anthracene	14500	600	550	700	30
Naphthalene*	2500	50	50	55	45 d.
Acetnaphthene	65000	14000	9000	10000	45
Fluorene	42000	12000	6500	6500	45
Anthracene	14500	150	175	160	45

*When fungal growth substrate is extracted prior to fungal growth and run on the G.C. using the PAH program, this peak occurs at the same time and magnitude as Naphthalene. Florosil does not totally remove it. All PAH analysis of soil/solid fungal inoculum mixtures indicate naphthalene at approximately 50 ppm. However it is unlikely that it is naphthalene in the soil. Additional analysis will be required to determine what this compound is.

Gas chromatography of the untreated control and of the 25 and 50% volume treatments after 45 days incubation was performed. Treated samples showed significant reductions in PAH concentration as indicated by the reduced number and area of the chromatographic peak.

Example 16

Degradation of polynuclear aromatic hydrocarbons (PAH) in water using cell-free extracts of *Phanerochaete chrysosporium*, sugar beet pulp cultures

Cultures of *P. chrysosporium* grown on sugar beet pulp were prepared as described in Example 1. Cultures were extracted by adding 2 volumes of water per one part weight of culture. The culture and water were blended for one minute, centrifuged, and filtered through a 0.8 micron filter. The cell-free, solids-free, filtrate contained 30.7 units per ml of Mn. Peroxidase activity as determined by phenol red assay. 20 ml samples of creosote contaminated water were dispensed to reaction vials. 0.5g, 2.0g, or 3.0g of culture extract was added to duplicate samples and the vials sealed. Three contaminated water samples were not mixed with culture extract. These samples were the controls. After 12 hours of incubation at room temperature, the controls and treated water samples were extracted and analyzed for PAH concentration using EPA method 610.

Concentrations of PAH in untreated and treated samples are shown below:

20 gram water samples; white-rot fungi - liquid enzyme extracts
12 hour treatment time
GC Analysis: EPA Method 610

Fungus Strain - P. chrysosporium

	Liquid enzyme dose			
	0	0.5g.	2.0g.	3.0g.
compound	concentration in micrograms/liter			
Acenaphthene	70	53.7	37.6	15.4
Fluorene	45	27	23.1	12.1
Phenanthrene	23	11.8	13.9	4.3

Example 17

Degradation of polynuclear aromatic hydrocarbons (PAH) in water using cell-free extracts of *Bjerkandera adusta*, sugar beet pulp cultures

Cultures of *B. adusta* grown on sugar beet pulp were prepared as described in Example 8. Cultures were extracted by adding 2 volumes of water per one part weight of culture. The culture and water were blended for one minute, centrifuged, and filtered through a 0.8 micron filter. The cell-free, solids-free, filtrate contained 95.1 units per ml of Mn. Peroxidase activity as determined by phenol red

assay. 20 ml samples of creosote contaminated water were dispensed to reaction vials. 2.0g or 5.0g of culture extract was added to duplicate samples and the vials sealed. Three contaminated water samples were not mixed with culture extract. These samples were the controls. After 12 hours of incubation at room temperature, the controls and treated water samples were extracted and analyzed for PAH concentration using EPA method 610.

Concentrations of PAH in untreated and treated samples are shown below:

20 gram water samples; white-rot fungi - liquid enzyme extracts
12 hour treatment time
GC Analysis: EPA Method 610

Fungus Strain - <u>B. Adusta</u>

	Liquid enzyme dose		
	0	2.0g.	5.0g.
compound	concentration in micrograms/liter		
Acenaphthene	70	70	0
Fluorene	45	31.6	31.6
Phenanthrene	23	25	26

Example 18

Degradation of PCB's Using Cultures of *Bjerkandera adusta* Grown on Sugar Beet Pulp

Polychlorinated biphenyls (PCB's) in soil were degraded by treatment with cultures of *B. adusta* grown on sugar beet pulp. PCB contaminated soil was obtained from an electric utility maintenance yard. The PCB's were a commercial mixture designated as Aroclor 1260. PCB type and concentration in soil was determined by extraction and gas chromatograph according to Environmental Protection Agency (EPA), method 8080. PCB analysis was performed by Mycotech Corporation (Butte, MT) and by independent, EPA certified laboratories.

Inoculum cultures of *B. adusta* CBS 595.78 were grown for 4 days at 28°C in an agitated flask in a nutrient solution of 10 g/l glucose, 5 g/l peptone and 3 g/l yeast extract. Sugar beet pulp was wetted to 70% moisture content with the same high nitrogen medium sterilized, cooled and inoculated at 10% volume with the inoculum culture. Inoculated sugar beet pulp was incubated for 10 days at 27°C with an airflow of 0.2 volumes air per volume of culture per minute with the air at approximately 90% relative humidity. At 10 days a sample of the culture was extracted by adding 3 volumes of water per volume of culture and homogenizing with a hand held blender for 20 seconds, centrifuging and filtering through a filter with a 0.8 micron pore size. The cell-free filtrate was assayed for the presence of peroxidase and manganese

peroxidase using phenol red and for oxidase using anis alcohol by standard procedures. Extracts contained 18.3 units per ml peroxidase and 99.5 units per ml manganese peroxidase and no oxidase activity at the time of application to soil.

Whole culture with a moisture content of 78% was mixed at 25% by volume with 50 grams of contaminated soil containing 45 ppm total PCB and the mixture placed in a covered glass bottle and incubated at room temperature for 30 days with periodic addition of water. Controls were prepared by treating contaminated soil with fungus culture that had been destroyed by autoclaving at 121°C for 20 minutes prior to addition to soil. After 30 days, treated and control soil samples were extracted and assayed for PCB concentration. Controls showed 45 ppm total PCB and treated samples 5 ppm total PCB. Gas chromatograph analysis showed degradation of all PCB congeners in the sample. Figures 1A and 1B are chromatographs of the control samples and treated samples showing uniform degradation of the PCB mixture.

Example 19

Degradation of PCB's Using Cultures of *B. adusta* Grown on Sugar Beet Pulp

PCB's in contaminated soil were degraded by treatment with cultures of *B. adusta* grown on sugar beet pulp. Cultures were grown and soil treated as described in Example 1 except that soil contamination was 330 ppm total PCB and equal volumes of whole wet culture and soil were used. After 30 days incubation

PCB concentration in the treated soil was 15 ppm with uniform reduction of all congeners in the PCB mixture. Figures 2A and 2B are chromatographs of extracts of control and treated soil samples.

Example 20

Degradation of PCB's in a Time Course Using a Slurry of *B. adusta*, Sugar Beet Pulp Cultures

B. adusta sugar beet pulp cultures were prepared as described in Example 1. After 10 days culture time, a slurry of the culture was prepared by adding 3 volumes of water per volume of wet culture. The mixture was homogenized in a blender. The resulting slurry contained 6.7% solids by weight. The slurry can be pumped or poured as a liquid for addition to soil or water. This slurry was stored in the refrigerator and used as the base stock for repeated addition of slurry.

The slurry as prepared contained 7.1 units per ml peroxidase activity and 76.4 units per ml Mn peroxidase activity by phenol red assay.

This experiment was designed as a time course using repeated applications of slurry to eight 50 gram duplicate soil samples. One of the soil samples was extracted without any slurry being added. This sample established the starting concentration. The other 7 soil samples had 50 grams of slurry added to them. After 7 days, all of these samples had approximately 50 grams of slurry added to them. Seven days later, another soil and slurry sample was extracted and analyzed for PCB's. The remaining 5 samples had

approximately 50 grams of slurry added. This process was repeated until 35 days had elapsed. No slurry was added to the remaining samples at 35 or 45 days. The results of the time course are summarized in the following table:

TIME COURSE
Slurry Application - *B. adusta*
PCB contaminated soil

Weight Inoculum	Elapsed Time	Concentration ppm
0 g.	0 days	325
50	7	236
100	14	122
140	19	66
275	26	35
315	35	20
335	45	12
335	55	less than 10

Example 21

The Use of B. adusta, Sugar Beet Pulp Culture Slurries to Degrade PCB's in a Field Demonstration

B. adusta sugar beet cultures were prepared as described in Example 1. After 10 days culture time, a slurry of the culture was prepared by adding 3 volumes of water to one volume of culture. This preparation was homogenized in a blender for one minute.

The slurry as prepared contained 10.3 units per ml peroxidase activity and 72.7 units per ml Mn peroxidase activity by phenol red assay.

Three soil plots approximately 46 cm in diameter with contamination extending to a depth of 15.5 cm were used for the field demonstration. These plots contained approximately 0.049 cubic meters of soil or 49 liters of soil. Eight liters of slurry were added to two of the plots. Seven days later, slurry was added to the third plot. Samples were taken before slurry addition, at 7 and 14 days. The results are shown in the following table:

Results of Field Demonstration
B. adusta, Sugar Beet Pulp Culture Slurry

	Initial Conc. ppm	7 days Elapsed Time	14 days Elapsed Time
Plot 1	410	370 ppm	330 ppm
Plot 2	260	230 ppm	210 ppm
Plot 3	260	230 ppm	

Example 22

The Use of B. adusta, Sugar Beet Pulp Cultures to Degrade PCB's in a Field Demonstration

B. adusta sugar beet pulp cultures were prepared as described in Example 1. The wet culture contained 18.3 units per ml peroxidase activity and 99.5 units per ml Mn peroxidase activity by phenol red assay.

Three soil plots measuring 2 meters x 3 meters with contamination extending 15.5 cm in depth were used for this field demonstration. Approximately 0.55 cubic meters of culture material were mixed into two of the plots. The third plot was treated 7 days later. The plots were sampled for PCB's prior to the addition of the fungus and again after 7 and 14 days elapsed time. The results are shown in the following table:

Results of Field Demonstration
B. adusta, Sugar Beet Pulp Culture

	Initial Conc. ppm	7 days Elapsed Time	14 days Elapsed Time
Plot 1	150	120 ppm	100 ppm
Plot 2	210	180 ppm	130 ppm
Plot 3	190	150 ppm	

Example 23

The Use of B. adusta, Sugar Beet Pulp Cultures to Degrade PCB's in a Field Demonstration Repeated Additions of B. adusta, Sugar Beet Pulp Cultures

B. adusta sugar beet cultures were prepared as described in Example 1. The initial wet culture contained 33.2 units per ml peroxidase activity and 85.9 units per ml Mn peroxidase activity by phenol red assay. Subsequent cultures were not assayed for enzyme activity.

Two soil plots approximately 46 cm in diameter with contamination extending to a depth of 15.5 cm were used for the field demonstration. These plots contained approximately 0.049 cubic meters of soil or 49 liters of soil. The whole culture was mixed 100% by volume with the soil. Samples were taken prior to addition of the whole culture and again after 12 days. After the 12 day sample, whole culture was again added to the plots at approximately 50% culture per volume of dirt. The plots were sampled 22 days later. Results of the sampling are shown in the following table. All analyses were performed by an EPA approved laboratory.

Results of Field Demonstration
B. adusta, Sugar Beet Culture
Two Applications

	Initial Conc. ppm	12 days Elapsed Time	34 days Elapsed Time
Plot 1	330	280 ppm	180 ppm
Plot 2	210	180 ppm	42 ppm

Example 24

The Use of B. adusta Sugar Beet Pulp Cultures to Degrade PCB's in a Field Demonstration

B. adusta sugar beet pulp cultures were prepared as described in Example 1. Two field soil plots at the site described in Example 1, measuring 46 cm diameter with contamination extending 15.5 cm deep were treated. The first plot contained a beginning PCB concentration of 220 ppm and the second plot 130 ppm. Plots were treated at the rate of 66% volume culture per volume of soil. After 34 days plots showed no evidence of culture substrate or cell mass. At 34 days plots were treated a second time at 70% volume with B. adusta sugar beet pulp cultures. Plots were assayed for PCB concentration by an EPA approved laboratory. Assay time intervals beginning from the first addition and PCB concentrations (ppm) are shown in the following table:

Results of Field Demonstration B. adusta Sugar Beet Pulp Culture, Two Applications

Elapsed Time Days After First Application	0	11	23	44	76	98
Plot 1	220	200	180	64	52	35
Plot 2	130	110	100	95	87	12

Example 25

The Use of P. chrysosporium, Sugar Beet Pulp Cultures to Degrade PCB's in a Field Demonstration Single Application of P. chrysosporium, Sugar Beet Pulp Cultures

Inoculum cultures of P. chrysosporium were grown for five days at 28°C in an agitated flask in a nutrient solution of 10 g/l glucose, 5 g/l peptone and 3 g/l yeast extract. Sugar beet pulp wetted to 70% moisture content with the same high nitrogen medium was autoclaved, cooled and inoculated at 10% volume with the inoculum culture. Inoculated sugar beet pulp was incubated for 7 days at 23°C with an airflow of .2 volumes air per volume of culture per minute with the air at approximately 90% relative humidity. At 7 days, a sample was extracted by adding 3 volumes of water per volume of culture and homogenizing with a hand held blender for 20 seconds, centrifuging and filtering through a filter with a 0.8 micron pore size. The cell free filtrate was assayed for the presence of peroxidase and manganese peroxidase using phenol red. Extracts contained 61 units per ml peroxidase and 64 units per ml manganese peroxidase.

Whole culture with a moisture of 75% was mixed at 25% by volume into a soil plot approximately 46 cm in diameter with contamination extending to a depth of 15.5 cm. The plot contained approximately 49 liters of soil. The soil was contaminated with a mixture of the Aroclors 1254 and 1260 with the majority of the contamination being Aroclor 1260. The soil pH was 8.5. Soil samples were taken at discrete intervals

and sent to an EPA approved laboratory for PCB analysis. The results are summarized in the following table:

Results of Field Demonstration
P. chrysosporium, Sugar Beet Pulp

Elapsed Time	Concentration in ppm
initial	200
11 days	190
19 days	180
50 days	170

Example 26

Degradation of PCB's Using Cultures of *P. chrysosporium* Grown on Sugar Beet Pulp

PCB's in contaminated soil were degraded by treatment with cultures of *P. chrysosporium* grown on sugar beet pulp. Cultures were grown as described in Example 8 except that the sugar beet pulp was wetted with the salts solution shown in the table below and grown for 6 days at 28°C. Duplicate 50 gram soil samples were prepared. Each sample was mixed with 150% by volume of whole wet fungal culture. The soil contained a mixture of the Aroclors 1242, 1254 and 1260 with 1254 and 1260 being the predominant types. The soil pH was 4.5.

The whole culture was assayed for manganese peroxidase and peroxidase activity as described in Example 8. The culture contained 76 units per ml of manganese peroxidase activity.

At discrete time intervals, a soil sample was sent to an EPA approved laboratory for PCB analysis. The results of those analyses are shown in the following table:

Degradation of PCB's Using
<u>P. chrysosporium</u> Cultures
Grown on Sugar Beet Pulp

Control	Elapsed Time 15 days	35 days	55 days
310 ppm	175 ppm	42 ppm	18 ppm

Typical Nutrient Solution Used

Substance	g/l	Substance	g/l
Glucose	10.0	$CaCl_2 \cdot 2H_2O$	.03
$NH_4H_2PO_4$	.05	Trace Elements	5 ml stock solution
KH_2PO_4	1.0	Veratryl Alcohol	0 or .14
$MgSO_4 \cdot 7H_2O$	1.0	Peptone	.05
		Yeast extract	.05

Example 27

Degradation of PCB's Using Cultures of *P. chrysosporium* Grown on Sugar Beet Pulp

PCB's in contaminated soil were degraded with treatments of *P. chrysosporium* grown on sugar beet pulp. Cultures were grown as described in Example 8 except that inoculum cultures were grown in a media containing .5g/l peptone, .5g/l yeast extract and 5g/l glucose. Duplicate 50 gram soil samples were prepared. The soil was contaminated with the mixture of Aroclors as described in Example 8. Different duplicate soil samples were mixed with 50%, 100% and 150% by volume wet fungal cultures.

The whole culture was assayed for manganese peroxidase and peroxidase activity as described in Example 8. The culture contained 66 per ml of manganese peroxidase activity.

The treated soil was analyzed for PCB's after 14 days. The results of those analyses are shown in the following table:

Degradation of PCB's Using *P. chrysosporium* Grown on Sugar Beet Pulp

Volume % Fungus	PCB Concentration After 14 days Elapsed Time
0% (control)	310 ppm
50%	230 ppm
100%	150 ppm
150%	101 ppm

Example 28

Degradation of PCB's Using Varying Rates of *P. chrysosporium* Sugar Beet Pulp Culture

P. chrysosporium was grown and used to treat 50 gram samples of PCB soil as described in Example 8. Identical soil samples were treated with different volumes of fungus culture and each treatment rate was sampled for PCB concentration at three different time intervals. Treatment rates were 25, 50, 100 and 150% volume of culture per volume of soil. Results are shown in the table below:

Vol % Fungus Added to Soil	Control	Elapsed Time in Days 15	35	55
0%	310			
25%	300	305	200	145
50%	No Value	270	190	130
100%	305	250	130	42
150%	No Value	175	42	18

NOTE: PCB concentrations in ppm

Example 29

Time Course of PCB Degradation Using *P. chrysosporium* Sugar Beet Pulp Cultures

P. chrysosporium was grown as described in Example 8 and used to treat identical 50 gram samples of the PCB contaminated soil also described in Example 8. Soil samples were treated with 150% volume of whole wet *P. chrysosporium* culture and incubated for 10 days. At 10 days an additional 50% volume of culture was added to one half of the 50 gram samples for a total of 200% volume treatment. Samples with 150 and 200% volume of culture were assayed for PCB concentration at 20, 30, 40 and 50 days elapsed time. Results are shown in the table below:

Elapsed Time in Days	Total Volume of Solid Inoculum	PCB Conc. in ppm
0	150%	332
10	150%	224
20	150%	154
20	200%	113
30	150%	83
30	200%	73
40	150%	31
40	200%	33
50	150%	13
50	200%	8

Example 30

Degradation of Pentachlorophenol Using Cultures of *P. chrysosporium* Grown on Sugar Beet Pulp

Pentachlorophenol (PCP) in soil was degraded by treatment with cultures of *P. chrysosporium* grown on sugar beet pulp. PCP was widely used as a wood preservative and is considered by the United States Environmental Protection Agency (EPA) to be a hazardous waste.

Two soil samples contaminated with different concentrations of PCP were obtained from a commercial laboratory. Sample 1 contained 8050 ppm and sample 2 contained 5246 ppm PCP.

P. chrysosporium sugar beet pulp cultures were prepared as follows: an inoculum culture was prepared by transferring *P. chrysosporium* maintained on nutrient agar slants to a sterile liquid medium containing 10 grams/liter sugar beet molasses, 2 grams/liter yeast extract and 1 gram/liter $KH_2\ PO_4$ adjusted to pH 3.5 with H_2SO_4. The liquid inoculum culture was incubated with agitation for four days at 30°C. Sugar beet pulp was wetted to 65% moisture content with water, autoclaved at 120°C, 05 psi for 20 minutes, cooled and inoculated at the rate of 10 ml inoculum culture per 100 ml volume of sugar beet pulp substrate. The inoculated sugar beet pulp was incubated for 7 days at 28°C with an airflow of .2 volume air per volume of culture per minute with the air at a relative humidity of about 90%.

Twenty-five (25) grams of contaminated soil was placed in 1-liter bottles and thoroughly mixed with either 25 grams or 50 grams of P. chrysosporium sugar beet pulp culture. Bottles with treated soil were loosely covered and incubated at 25°C for 21 days. After 21 days, soil was analyzed for PCP concentration by a modification of EPA method 8040. The entire contents of each treatment bottle - fungus culture and contaminated soil - was transferred to a soxhlet apparatus and extracted for eight hours with hexane. The extract was concentrated and analyzed by gas chromatography. Concentration was determined by comparison with standards of known PCP concentration. For experimental controls, 25 grams of contaminated soil was treated with wetted, sterile sugar beet pulp without fungus growth. Results of PCP assays for fungus treated and control treatments are shown in Table 1.

Table 1

Pentachlorophenol Degradation
Soil #1

As Measured: 8050 ppm

Treatment	Conc. After Treatment	% Remaining
WRF#1 Control 25g	7,040 ppm	87.5
WRF#1 Treated 25g	3,810	47.3
WRF#1 Control 50g	5,230	65.0
WRF#1 Treated 50g	1,310	16.3
WRF#1 Control 25g	3,200	59.0
WRF#1 Treated 25g	2,466	45.4
WRF#1 Control 50g	3,801	70.1
WRF#1 Treated 50g	1,456	26.8

Example 31

Degradation of Pentachlorophenol Using Cultures of *B. adusta* Grown on Sugar Beet Pulp

PCP in soil was degraded by treatment with cultures of *B. adusta* grown on sugar beet pulp. Soil samples were the same as those described in Example 30.

B. adusta sugar beet pulp cultures were prepared as described in Example 30, except that *B. adusta* was used.

Soil was treated with *B. adusta* sugar beet cultures and analyzed for PCP concentration as described in Example 30.

Results are shown below:

Table 2

Pentachlorophenol Degradation
Soil #2

As Measured: 5426 ppm

Treatment	Conc. After Treatment	% Remaining
WRF#2 Control 25g	6,961 ppm	86.5
WRF#2 Treated 25g	6,295	78.2
WRF#2 Control 50g	7,233	89.6
WRF#2 Treated 50g	5,392	67.0
WRF#2 Control 25g	4,820	88.8
WRF#2 Treated 25g	4,016	74.0
WRF#2 Control 50g	4,603	84.8
WRF#2 Treated 50g	4,602	84.8

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims

1. A method of growing white-rot fungus, comprising growing the fungus under growth-supportive conditions on a sugar beet pulp substrate.

2. A method of claim 1, wherein the white-rot fungus is selected from the group of genera consisting of *Phanerochaete*, *Phlebia*, *Trametes*, *Pleurotus* and *Bjerkandera*.

3. A method of claim 1, wherein the sugar beet pulp is mixed with straw.

4. A method of claim 1, wherein growth-supportive conditions sufficient to support primary metabolic growth of the fungus are maintained substantially throughout the growing period.

5. A method of claim 1, further comprising the step of separating lignin-degrading enzymes from the culture substrate after a desired period of growth on the substrate.

6. A method of claim 1, for producing a by-product of fungal growth, further comprising the step of isolating a by-product of fungal growth from the culture of fungus.

7. A method of claim 6, wherein the by-product is an aromatic-compound degrading enzyme.

8. A method of claim 7, wherein the aromatic-compound degrading enzyme is a lignin-degrading enzyme.

9. A method of claim 8, wherein the enzyme is selected from the group consisting of peroxidases, manganese peroxidases, oxidases and laccases.

10. A method of cultivating white-rot fungus, comprising the steps of:
   a) mixing an inoculum of white-rot fungus with a substrate of sugar beet pulp having an absorbed water content of 40-80%;
   b) growing white-rot fungus on the sugar beet pulp substrate at a temperature between 20-40°C;
   c) aerating the inoculated substrate at a rate of between .05 to 20 volumes of air per minute per volume of substrate during the growing period with air having an oxygen level above 7% and a relative humidity of 70-99%.

11. A method of claim 10 in which water is used to wet the sugar beet pulp.

12. A method of claim 11 in which a solution containing glucose and protein is used to wet the sugar beet pulp.

13. A method of claim 10, further comprising the steps of adding water to the substrate after the growing period and then centrifuging and filtering the mixture to separate a solution of cell-free enzymes from the substrate.

14. A method of claim 13, wherein the filtering step is carried out with filters having a screen mesh of no larger than 0.8 micron.

15. A method of claim 10, wherein the white-rot fungus is selected from the group consisting of Phanerochaete chrysosporium, Phlebia tremellosus, Trametes versicolor, and Bjerkandera adusta.

16. A method of claim 10, wherein enzyme production occurs during the primary metabolic growth phase.

17. A method of claim 10, wherein, prior to step A, the sugar beet pulp is sterilized by autoclaving and cooled to between 20-40°C.

18. A method of claim 10, wherein straw is added to the sugar beet pulp substrate prior to step B.

19. A method of producing lignin-degrading enzymes, comprising growing white-rot fungus on a sugar beet pulp substrate and recovering lignin-degrading enzymes produced by the fungus.

20. A method of claim 19, wherein the white-rot fungus is selected from the group of genera consisting of Phanerochaete, Phlebia, Trametes, Pleurotus and Bjerkandera.

21. A method of claim 19, wherein growth-supportive conditions sufficient to support primary metabolic growth of the fungus are maintained substantially throughout the growing period.

22. A fungal culture comprising a ligninase-producing white-rot fungus in admixture with and grown on a solid substrate comprising sugar beet pulp.

23. The culture of claim 22, wherein the white-rot fungus is selected from the group of genera consisting of Phanerochaete, Phlebia, Trametes, Pleurotus and Bjerkandera.

24. A bioremediation method for degrading aromatic contaminants in soil or water, the method comprising the step of

mixing with the soil or water containing said aromatic contaminant a fungal culture comprising a ligninase-producing white-rot fungus in admixture with a solid substrate comprising sugar beet pulp or an essentially cell-free, enzyme-containing extract of the fungal culture at a concentration sufficient and at a temperature sufficient to degrade enzymatically at least a portion of the aromatic contaminant in the soil or water.

25. The method of claim 24, wherein the white-rot fungus is selected from the group of genera consisting of Phanerochaete, Phlebia, Trametes, Pleurotus and Bjerkandera.

26. The method of claim 24, wherein the aromatic contaminant is a chlorinated aromatic compound or a polynuclear aromatic hydrocarbon.

27. A bioremediation method for degrading aromatic contaminants in soil or water, the method comprising the step of

mixing with the solid or water containing said aromatic contaminant a fungal culture comprising a ligninase-producing white-rot fungus in admixture with a solid substrate comprising sugar beet pulp at a concentration sufficient and at a temperature sufficient to degrade enzymatically at least a portion of the aromatic contaminant in the soil or water.

28. The method of claim 27, wherein the white-rot fungus is selected from the group of genera consisting of Phanerochaete, Phlebia, Trametes, Pleurotus and Bjerkandera.

29. The method of clam 28, wherein the white-rot fungus is selected from the group consisting of P. chrysosporium and B. adjusta.

30. The method of claim 27, wherein the aromatic contaminant is a chlorinated aromatic compound or a polynuclear aromatic hydrocarbon.

31. A bioremediation method for degrading chlorinated aromatic compound or a polynuclear aromatic hydrocarbon in soil or water, the method comprising the step of

mixing with the solid or water containing the aromatic contaminant a fungal culture comprising ligninase-producing P. chrysosporium in admixture with a solid substrate comprising sugar beet pulp at a concentration sufficient and at a temperature sufficient to degrade enzymatically at least a portion of the chlorinated aromatic compound or a polynuclear aromatic hydrocarbon in the soil or water.

32. A bioremediation method for degrading chlorinated aromatic compound or a polynuclear aromatic hydrocarbon in soil or water, the method comprising the step of

mixing with the solid or water containing the aromatic contaminant a fungal culture comprising ligninase-producing B. adjusta in admixture with a solid substrate comprising sugar beet pulp at a concentration sufficient and at a temperature sufficient to degrade enzymatically at least a portion of the chlorinated aromatic compound or a polynuclear aromatic hydrocarbon in the soil or water.

33. A method of degrading a polyhalogenated biphenyl compound in a material, comprising contacting the material with a culture of white-rot fungus grown on a sugar beet pulp substrate under conditions sufficient to degrade the polyhalogenated biphenyl compound in the material.

34. A method of claim 33, wherein the polyhalogenated biphenyl compound is a polychlorinated biphenyl.

35. A method of claim 33, wherein the material is soil or water contaminated with a polyhalogenated biphenyls.

36. A method of claim 33, wherein the white-rot fungus is selected from the genus Phanerochaete or Bjerkandera.

37. A method of claim 36, wherein the white-rot fungus is Phanerochaete chrysosporium.

38. A method of claim 36, wherein the white-rot fungus is Bjerkandera adusta.

39. A method of degrading a polychlorinated biphenyl compound in contaminated soil, comprising mixing the soil with a solid state fungal culture comprising Bjerkandera adusta in admixture with a substrate of sugar beet pulp, under conditions sufficient to degrade the polychlorinated biphenyl in the soil or water.

40. A method of degrading a polychlorinated biphenyl compound in contaminated soil, comprising mixing the soil with a solid state fungal culture comprising Phanerochaete chrysosporium in admixture with a substrate of sugar beet pulp, under conditions sufficient to degrade the polychlorinated biphenyl in the soil or water.

1/2

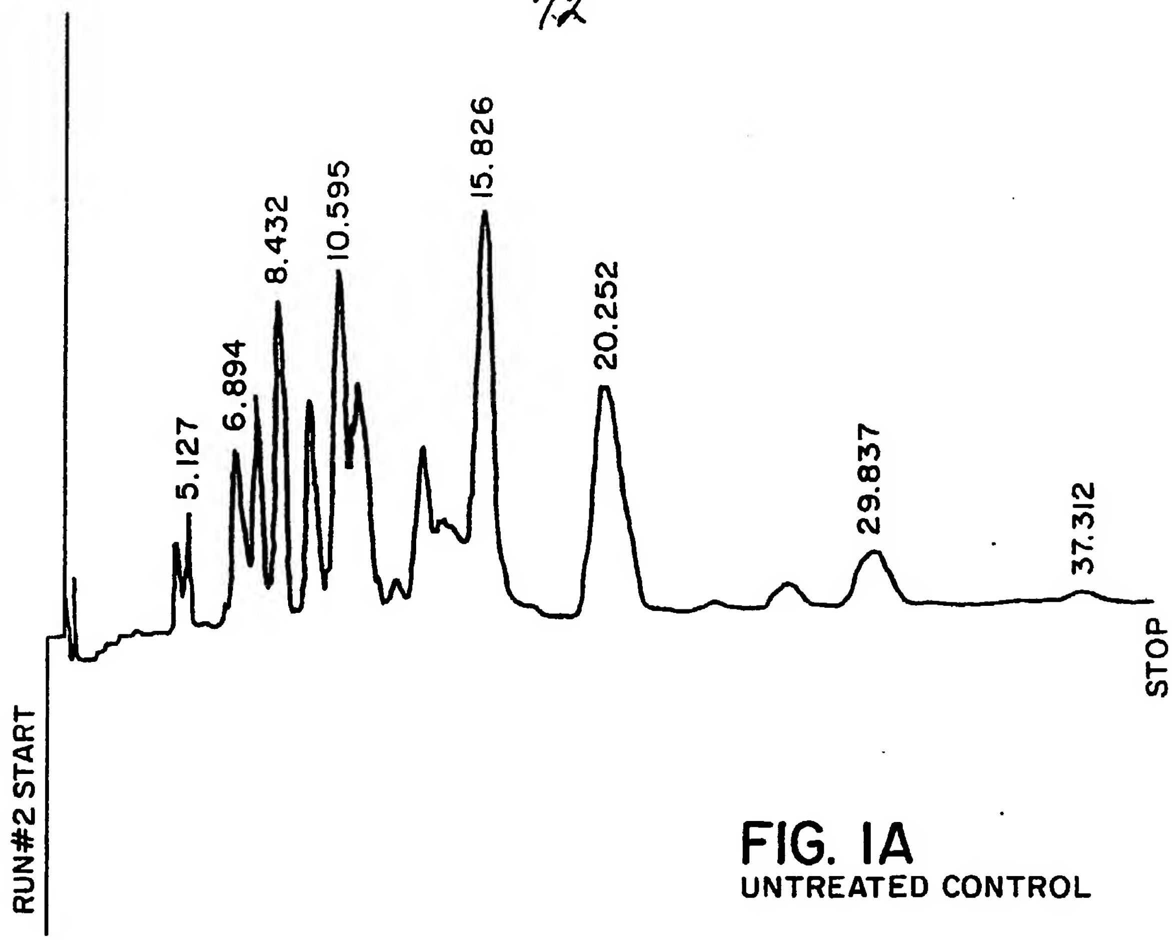

FIG. 1A
UNTREATED CONTROL

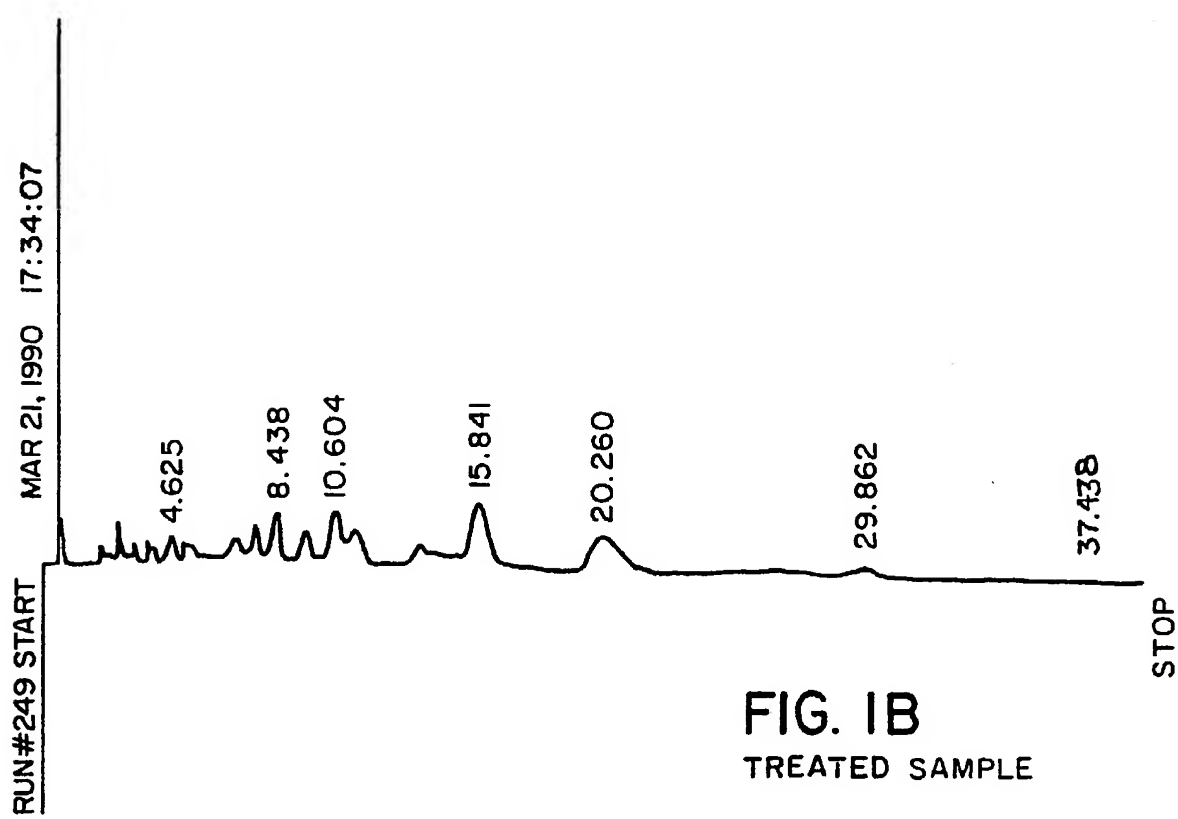

FIG. 1B
TREATED SAMPLE

300 PPM CONTROL WITHOUT INOCULUM

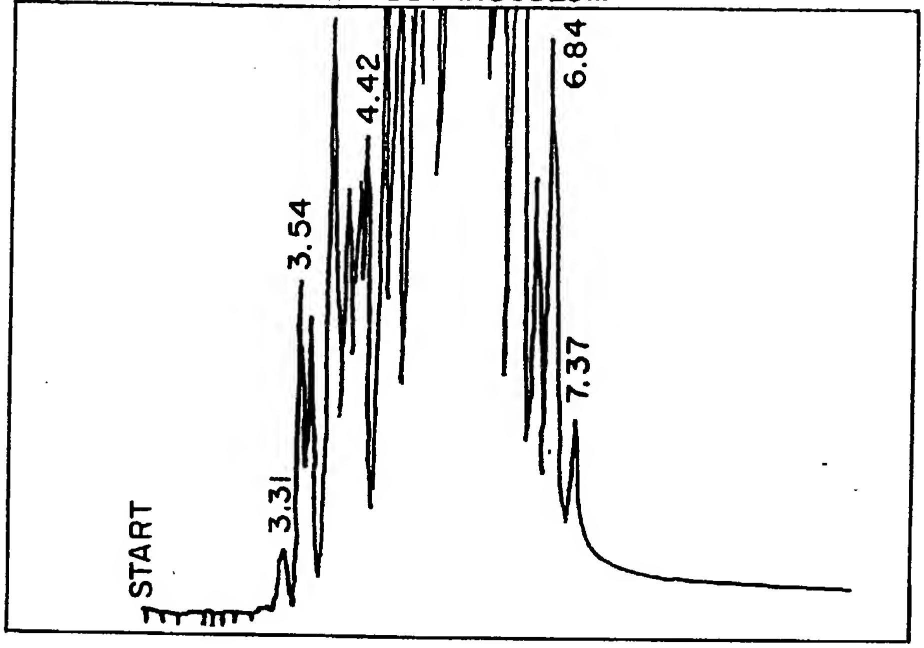

FIG. 2A

300 PPM SOIL INOCULATED WITH B. ADUSTA

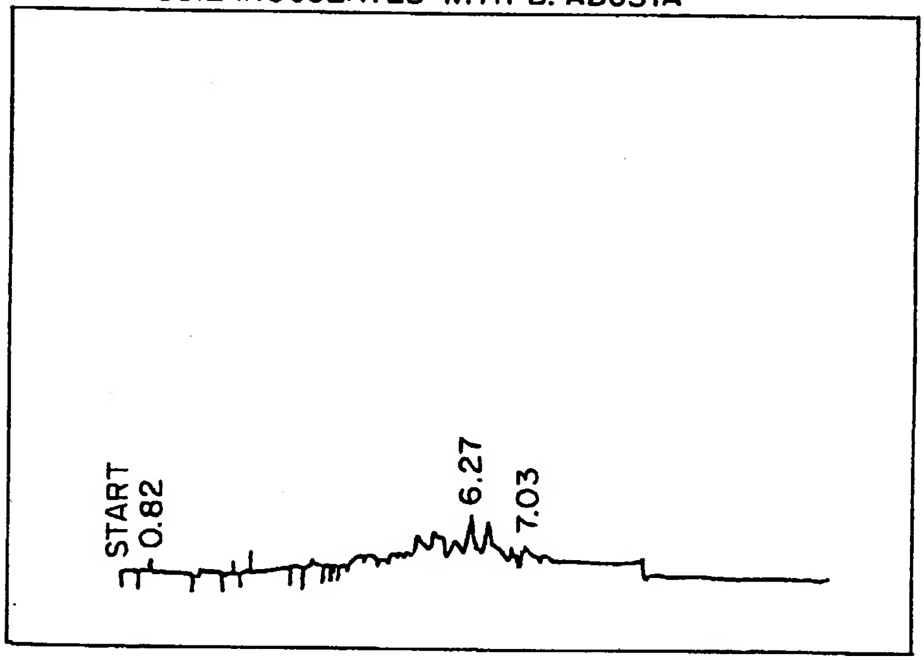

FIG. 2B

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00871

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): Please See Attached Sheet.
US CL : Please See Attached Sheet.

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/166, 171, 195, 252, 254, 262, 262.5, 264; 47/1.1; 162/9, 72; 210/606, 610, 611, 632

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS, DIALOG

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	Mic. Ital., Volume 2, 1984, Floriano Ferri, "Growth Tests of Polyporus tuberaster", pages 2-6, see the abstract.	1-40
Y	US, A, 4,803,800 (Romaine et al.) 14 February 1989, see entire document.	1-40
Y	US, A, 4,554,075 (Chang et al.) 19 November 1985, see entire document.	1-40
Y	Applied and Environmental Microbiology, Volume 55, issued January 1989, John A. Bumpus, "Biodegradation of Polycyclic Aromatic Hydrocarbons by Phanerochaete chrysosporium", pages 154-158, see entire document.	1-40
Y	US, A, 4,891,320 (Aust et al.) 02 January 1990, see entire document.	27-40
Y	Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications, Volume 1, issued 1980, (CRC Press), T. Kent Kirk, et al., "The Chemistry of Lignin Degradation by White Rot Fungi", page 215, see Introduction.	1-26

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
18 MAY 1992	[illegible]
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	DEBORAH K. WARE

Form PCT/ISA/210 (second sheet)(May 1986) B

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Applied Microbiology and Biotechnology, Volume 21, 1-40, issued 1985, Agosin et al., "Solid-state fermentation, lignin degradation and resulting digestibility of wheat straw fermented by selected White Rot Fungi", pages 397-403, see entire document.	1-40

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone Practice) (Telephone Practice)
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12P 5/00; C12N 1/22, 1/14; C07C; D06M 16/00; C12N 9/14; C12P 1/02; A01G 1/04; D21C 9/00; D21C 3/20; C02F 3/00, 1/00

I. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/166, 171, 195, 252, 254, 262, 262.5, 264; 47/1.1; 162/9, 72; 210/606, 610, 611, 632

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. a) Claims 1-9 drawn, to a first method of making White Rot Fungi, Class 435, Subclass 264.
b) Claims 19-21, drawn to a first method of using White Rot Fungi, Class 435, Subclass 262.
c) Claims 22-23, drawn to a first product including the White Rot Fungi, Class 435, Subclass 171.

II. Claims 10-18, drawn to a second method of making White Rot Fungi, Class 210, 606.

III. Claims 24-26, drawn to a second method of using White Rot Fungi, Class 210, Subclass 611.

IV. Claims 27-32, drawn to a third method of using White Rot Fungi, Class 47, Subclass 1.1.

The claims of groups I, II, III, and IV are drawn to distinct methods and a product produced by the first method of making. Each have a separate status in the art as shown by their different classification. First method is to grow a white rot fungi first product while the second method includes the action steps of cultivating it. PCT Rules 13.1 and 13.2 do not provide for multiple distinct methods within a single general inventive concept.